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Enhancement of TAT-Induced Transactivation of the HIV-1 LTR by Two Genomic Fragments of HHV-6

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Clinical and experimental observations suggest that human herpesvirus-6 (HHV-6), a T-lymphotropic herpesvirus, may act as a cofactor in the acquired immunodeficiency syndrome (AIDS). Moreover, a possible role of HHV-6 in the increased incidence and severity of cervical carcinoma in human immunodeficiency virus (HIV)-infected women was suggested by the recent observation that HHV-6 can infect cervical carcinoma cells, accelerating their tumorigenicity in vivo. Therefore, the ability of four HHV-6 genomic clones derived from HHV-6 to transactivate the long terminal repeat (LTR) of HIV-1 in two cervical carcinoma cell lines and in a T-lymphoid cell line was tested. Two HHV-6 clones, pZVH-14 and pZVB-70, which were previously shown to increase the expression of human papillomavirus (HPV)-transforming genes, were, per se, weak transactivators of the HIV-1 LTR. However, an increased effect occurred when these clones were combined with the HIV-1 transactivator TAT-1. No such effect was seen with two other HHV-6 clones used as controls. Analysis with HIV-1 LTR deletion mutants indicated that this enhancing effect requires the presence of elements contained in both the enhancer region and the TAT activation region (TAR) of HIV-1. This data may have implications for the potential role of HHV-6 in AIDS and AIDS-related cervical carcinoma.

KEY WORDS: cervical carcinoma, AIDS, papillomavirus

INTRODUCTION

Human herpesvirus-6 (HHV-6) was isolated originally in 1986 from the peripheral blood of patients with lymphoproliferative disorders and AIDS (Salahuddin et al., 1986). In vitro and in vivo studies have demonstrated that HHV-6 has a predominant tropism for CD4+ T lymphocytes [Lusso et al., 1988; Takahashi et al., 1989] and exerts a marked cytopathic effect on them [Salahuddin et al., 1986; Lusso et al., 1988]. The ubiquitous nature of HHV-6 [Saxinger et al., 1988] has hampered the use of seroepidemiologic probes to establish a firm association with disease. To date, the only disease that has been conclusively linked to HHV-6 is exanthem subitum [Yamanishi et al., 1988], a febrile illness associated with primary infection in infants. Recent evidence indicates that HHV-6 infection is frequently reactivated in immunocompromised hosts, such as post-transplant patients [Okuno et al., 1990; Carrigan et al., 1991; Yoshikawa et al., 1991; Cone et al., 1993] and human immunodeficiency virus (HIV)-infected individuals [Corbellino et al., 1993; Knox et al., 1994]. A series of clinical and experimental observations indicates that HHV-6 may be a cofactor in the progression of HIV infection toward full-blown AIDS [Lusso et al., 1994]. Like other herpesviruses HHV-6 was shown to transactivate the expression of the HIV-regulatory elements contained in the viral long terminal repeat (LTR) [Lusso et al., 1989; Ensoli et al., 1989; Horvat et al., 1989; Geng et al., 1992; Martin et al., 1991; Wang et al., 1994]. By using HIV-LTR deletion mutants, the region of the LTR responsive to HHV-6 transactivation was mapped to the NFκB sites in the enhancer element of HIV-1 [Ensoli et al., 1989]. In the case of HHV-6, however, such transactivating ability may be biologically significant because this virus can coinfect CD4+ T cells with HIV [Lusso et al., 1989] and, thereby, has the possibility to directly trigger or enhance the replication of HIV in vivo.

Recent studies indicate that HIV-infected women have a higher risk for genital cancer than HIV-negative women prompting the Center for Disease Control and Prevention to include cervical cancer within the list of AIDS-defining diseases [1992]. Furthermore, the progression of cervical intraepithelial neoplasia may be accelerated in the course of HIV infection [Maiman et al., 1990; Vermund et al., 1991]. The mechanism whereby HIV can contribute directly or indirectly to the development of cervical cancer is presently unknown. Although infection with human papillomaviruses (HPVs) has been

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associated with the development of cervical cancer, only a low frequency of HPV-positive women develop neoplasias, suggesting the importance of cofactors [Schiffman, 1992]. A possible role of HHV-6 in cervical cancer was suggested by the recent observation that HHV-6 infects genital epithelial cells, transactivates HPV-transforming genes, and induces accelerated tumorigenesis in vivo [Chen et al., 1994a]. Furthermore, HHV-6 sequences were detected by polymerase chain reaction (PCR) and *in situ* hybridization in nuclei of epithelial cells of HPV-positive cervical carcinomas [Chen et al., 1994b]. These observations suggest that a complex network of viral interactions, involving HIV, HHV-6, and human papillomaviruses (HPVs), may occur in HIV-infected women.

The current study demonstrates that HHV-6 genomic fragments transactivate the regulatory sequences of HIV-1 in cervical epithelial and T-cell lines. The gene products from two HHV-6 molecular clones (pZVH-14 and pZVB-70), found previously to transactivate the expression of the HIV-1 LTR [Wang et al., 1994; Horvat et al., 1991] and HPV-transforming genes [Chen et al., 1994a], have minimal effect on the HIV-1 LTR in the experimental model used but significantly enhance the effect of the HIV-1 transactivator, TAT.

MATERIALS AND METHODS

The HIV-1 constructs used were pC15-CAT (HIV-LTR-CAT) [Arya et al., 1985], which contains the complete HIV-1 LTR linked to the CAT reporter gene; CD23-CAT (−117/+80) and CD54-CAT (−48/+80), which are sequential deletions of the 5′ end of pC15-CAT plasmid in the U3 region of HIV-1 3′ LTR [Heisig et al., 1987]; and BS-CAT, a 3′ deletion mutant from the Bgl II site (+25) to the Sst I site (+38) of the CD23 plasmid. pTAT contains the TAT gene of HIV-1 [Ensoli et al., 1989]. The pZVB-70 fragment [Josephs et al., 1988], a 22.3-kb clone of the HHV-6 genome, contains the GGGTTA tandem repeat [Kishi et al., 1988] and a G protein-coupled receptor homologue [Berneman et al., 1992]. The pZVH-14 clone [Josephs et al., 1986] has an 8.7-kb HHV-6 insert that contains an open reading frame for the putative large tegument protein of HHV-6 [Josephs et al., 1991]. pZVB-43 [Josephs et al., 1991] is an 11-kb HHV-6 clone that includes an open reading frame for the glycoprotein-H molecule [Josephs et al., 1991]. The pZVB-9 clone [Josephs et al., 1988] contains an 11.0-kb HHV-6 insert coding for the major DNA-binding protein of HHV-6 [Berneman et al., 1992]. The vectors for pZVB-70 and pZVH-14 are pBluescript and pIBI-31, respectively.

HeLa and C-33A are HPV-18-positive and HPV-negative cervical carcinoma cell lines, respectively. Jurkat is a neoplastic CD4+ T-cell line. The cells were transfected with pC15-CAT or deletion mutants alone or with pTAT as negative and positive controls, respectively. The HIV-1 LTR pC15-CAT plasmid or mutants were also cotransfected with different molecular clones of HHV-6, pZVB-70, pZVH-14, pZVB-43, or pZVB-9 in the presence or absence of pTAT. Transfection of cervical cells was performed using a lipofection reagent according to the manufacturer's procedure (GIBCO BRL, Bethesda, MD). Briefly, 10 μg of DNA, each in 50 μl of TE, were mixed with 1 ml of F12/DMEM medium containing 50 μg of lipofection reagent in a total of 1.1 ml, and added to subconfluent HeLa or C-33A cells. Cells were incubated for 2 hours at 37°C with shaking at 15-minute intervals. The next day cultures were washed with a phosphate-buffered saline and incubated in fresh F12/DMEM with 5% fetal bovine serum. After 2 additional days of incubation, the transfected cells were harvested. In experiments with T cells, plasmid DNA (15 μg) was transfected by electroporation with a single pulse of 960μFD, 250 V from a Gene Pulser apparatus (Bio-Rad, Richmond, CA). Cellular extract was prepared by sonication, followed by centrifugation to remove cell debris. CAT assays using (14C) chloramphenicol and thin-layer chromatography have been previously described in detail [Gorman et al., 1982]. The data obtained using HeLa cells was also analyzed using different aliquots of protein obtained by serial dilutions. The degree of conversion was determined by recovering the acetylated and non-acetylated forms of chloramphenicol, followed by counting in a liquid scintillation counter.

RESULTS

Two HHV-6 genomic clones, pZVH-14 and pZVB-70, were studied with pTAT for their ability to transactivate the HIV-1 LTR. The degree of transactivation of plasmid pC15-CAT, containing the complete HIV-1 LTR in HeLa cells after cotransfection with either pZVH-14 or pZVB-70 (Fig. 1, mean of four experiments ± S.E.) was 1.5 and 1.9 folds, respectively. Similar results were obtained in a human CD4+ T-lymphoid line, Jurkat, in which pZVH-14 and pZVB-70 induced a 2.1- and 2.2-fold transactivation, respectively. However, pZVH-14 and pZVB-70 markedly increased the effect of pTAT in inducing transactivation of the HIV-1 LTR. Whereas pTAT alone increased CAT activity 62 fold over the pC15-CAT control, pTAT in combination with either pZVH-14 or pZVB-70 augmented the CAT activity 95 and 120 fold (Fig. 1), respectively, thus resulting in an enhancement of the transactivation of the HIV-1 LTR induced by pTAT. In contrast, no significant activity over the level of the controls occurred with the other two HHV-6 genomic clones, pZVB-9 and pZVB-43, used either alone or in combination with pTAT (data not shown). These two clones had also failed to transactivate the HPV-18 promoter in HeLa cells [Chen et al., 1994a]. Similar results were obtained with Jurkat and another cervical cell line, C-33A (Fig. 1). With Jurkat cells the CAT enhancement with pTAT only, pTAT plus pZVH-14, and pTAT plus pZVB-70 was 43, 65, and 80 fold, respectively. No transactivation occurred in Jurkat cells when either pZVB-9 or pZVB-43 was cotransfected with pC15-CAT with or without TAT (data not shown). In the C-33A cell line, despite the low level of pC15-CAT activation induced by pTAT, a significant enhancement was seen with pZVH-14 and pZVB-70 clones. The levels of transactivation

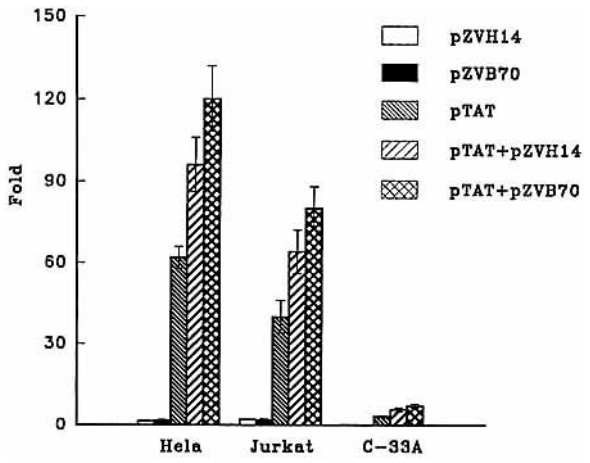

Fig. 1. Transactivation of the HIV-1 LTR linked to the CAT gene by pTAT plus either pZVH-14 or pZVB-70 in cervical epithelial cells and T cells. The fold activation was calculated by the ratio between the percent conversion obtained with pTAT plus either pZVH-14 or pZVB-70 and the percent conversion with pC15-CAT only. Results from four different experiments are expressed as mean ± standard error.

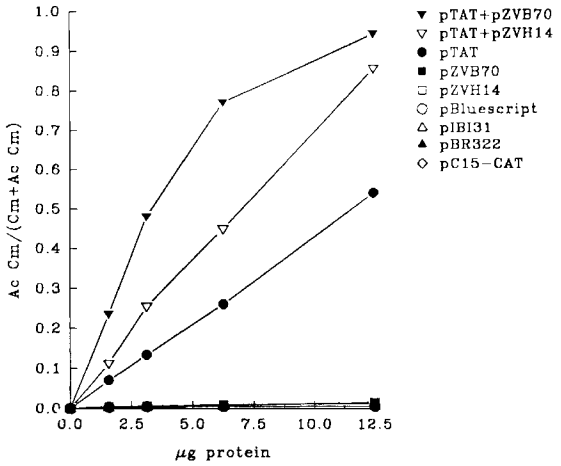

Fig. 2. Comparative transactivation of the HIV-1 LTR (pC15-CAT) by two molecular clones of HHV-6 in the presence or absence of pTAT in HeLa cells. Different amounts of protein from each sample were incubated for 45 minutes in a CAT assay. Quantitations of the CAT assay were obtained by measuring the conversion of 14C-chloramphenicol (Cm) to acetylated chloramphenicol (AcCm), as previously described [Gorman et al., 1982].

were 3.2 folds with pTAT alone, 5.7 with pTAT plus pZVH-14, and 7.0 with pTAT plus pZVB-70. The relatively low levels of activation obtained with C-33A compared to HeLa and Jurkat cells are reproducible and probably reflect the difference in growth rate which was *approximately* one third compared to the other two cell lines.

The level of transactivation induced by the HHV-6 clones in HeLa cells was precisely quantitated by titrating the protein content (Fig. 2). Irrespective of the amount of protein used, ranging from 1.5 to 12.5μg, no significant transactivation occurred with either of the two molecular clones alone. In the range between 1.5 and 6 μg of protein, an exponential increase in transactivation occurred with pTAT plus either pZVB-70 or pZVH-14, compared to pTAT alone. At 1.5 μg of protein, an increase of 12 and 24 fold was obtained in pTAT plus pZVH-14 and pTAT plus pZVB-70, respectively. At a protein concentration of 12.5 μg, a distinct separation between pTAT alone and pTAT combined with either of the two HHV-6 clones still existed; however, pTAT plus pZVB-70 began to plateau after 6 μg of protein.

To identify the functional regions within the HIV-1 LTR responsible for the increased transactivation induced by HIV-1 TAT and the HHV-6 clones pZVH-14 and pZVB-70, three LTR deletion mutants were tested in HeLa cells (Fig. 3). The first two mutants tested, CD23 (−117/+80) and CD54 (−48/+80), were 5′ deletion mutants of the LTR. The CD23 mutant contains the major enhancer elements of the HIV-1 LTR; e.g., Sp1- and NFκB-binding sites which are lacking in CD54. Transactivation increased by pTAT and either pZVH-14 or pZVB-70, occurred with the CD23 but not with the CD54 mutant (Fig. 3). Another mutant BS has a deletion (from +25 to +38) in the TAT-activating region TAR, which is necessary for the TAT response [Heisig et al., 1987; Rosen et al., 1985]. No transactivation was seen with this construct, irrespective of the presence of the HHV-6 clones. Thus, both the TAR and the enhancer motifs of the HIV-1 LTR are required for the cooperative effect of TAT and the HPV and HHV-6 genomic clones, pZVH-14 and pZVB-70. These results agree with previous observations utilizing deletion mutants of the HIV-1 LTR in HHV-6 in a HHV-6 infected T-cell line [Ensoli et al., 1989].

DISCUSSION

Several reports have demonstrated that the HIV-1 LTR is transactivated by HHV-6 infection, as well as by HHV-6 genomic fragments [Lusso et al., 1989; Ensoli et al., 1989; Horvat et al., 1989; Geng et al., 1992; Martin et al., 1991; Wang et al., 1994; Horvat et al., 1991]. Both major HHV-6 subgroups A and B exert this effect [Horvat et al., 1991; Garzino-Demo, unpublished results]. The present study demonstrates an enhanced transactivation of the HIV-1 LTR by TAT in combination with two HHV-6 genomic clones, suggesting that once initiated HIV-1 replication can be amplified in cells coinfected by HHV-6 and HIV-1. These results are consistent with an additive-transactivating effect previously documented in human T cells coinfected by HHV-6 and HIV-1 compared to the same cells infected with HIV-1 only [Ensoli et al., 1989; Geng et al., 1992]. This may be explained by the fact that the major HHV-6-responsive elements within the HIV-1 LTR are different from the TAT-response element, TAR [Ensoli et al., 1989].

The current data differ from that of Horvat et al. [1991], who reported that the pZVB-70 and pZVH-14 genomic clones as well as a third clone pZVB-10, efficiently transactivate the HIV-1 LTR in the absence of TAT in normal human peripheral blood T cells. It was found that pZVH-14 and pZVB-70 are, per se, very ineffi-

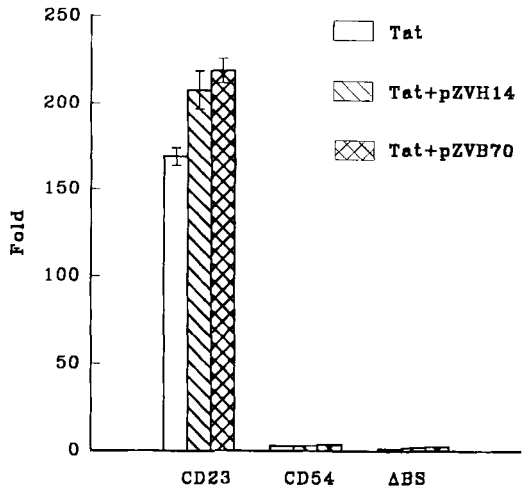

Fig. 3. Transactivation of HIV-1 LTR deletion mutants linked to the CAT gene in HeLa cells. CD23 CAT (−117/+80) and CD54 CAT (−48/+80) are deletion mutants at the 5′ end of pC15-CAT plasmid (4); BS CAT is a 3′ deletion mutant from the Bgl II site (+25) to the Sst I site (+38) of the CD23 plasmid. CAT assays were performed with 10 μg of cell lysate for 45 minutes. The fold increase of CAT activity was calculated from the ratio between the percent conversion with pTAT plus the HHV-6 molecular clones and the percent conversion with pC15-CAT alone. Results are means ± standard error from four different experiments.

cient transactivators of the HIV-1 LTR. Quantitative differences between this and Horvat's study may be related to the different cell types and methodologies used.

The data extends the observations previously obtained with human T lymphoblasts, CV1, and Vero cells [Wang et al., 1994; Horvat et al., 1991] to cervical epithelial cells. It is now well accepted that HIV-infected women have higher rates of recurrence and death from cervical cancer as opposed to HIV-negative women [Maiman et al., 1990; Feingold et al., 1990; Mandelblatt et al., 1992]. However, the mechanism for this phenomenon is still unknown. The recent demonstration that HHV-6 can infect both neoplastic and *in vitro* HPV-transformed cervical epithelial cells, inducing an accelerated tumorigenic ability in nude mice [Chen et al., 1994a], suggests a possible role of HHV-6 in the progression of cervical carcinoma. This hypothesis is strengthened by the detection of HHV-6 genetic sequences in some cervical carcinomas [Chen et al., 1994b]. Thus, it is possible that a complex network of interactions may occur in HIV-infected women between HIV itself, HHV-6, which has been suggested as a cofactor in AIDS [Lusso et al., 1994], and a subset of HPVs which are strongly linked to the etiology of cervical cancer. In this respect it is particularly intriguing that the two HHV-6 clones (pZVH-14 and pZVB-70) which synergize with TAT also transactivated the expression of HPV-transforming genes [Chen et al., 1994a]. Precise identification of the transactivation-associated genes contained in these genomic fragments of HHV-6 may be important for the dissection of the potential mechanisms of interaction between HHV-6, HPV, and HIV in human genital cells.

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